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The genetics of osteoarthritis in STR/ort mice[1]

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Summary

Objective: The complex genetics of osteoarthritis (OA) are still poorly defined. To circumvent the problems of genetic and environmental diversity hampering the analysis in humans, we investigated quantitative trait loci (QTL) associated with murine OA in the STR/ort strain which spontaneously develops osteoarthritic changes of the knee joints, overweight and elevated serum cartilage oligomeric matrix protein (COMP) levels.

Methods: Two hundred and seventy six male F2 intercross (STR/ort × C57BL/6) animals were genotyped using 96 microsatellite markers and phenotyped by analyzing weight, serum COMP levels and osteoarthritic changes of the knee joints. Quantitative trait analyses were performed using the R/qtl software.

Results: Elevated weight, serum COMP levels and osteoarthritic changes of the knee joints in the F2 generation compared to C57BL/6 parental animals confirm Mendelian inheritance. Quantitative trait analyses revealed three weight-, one serum COMP- and one OA-locus.

Conclusions: The weight-QTL coincide with previously described genes and QTL involved in fatty acid metabolism and offer a plausible explanation for the observed phenotype in STR/ort mice. The exact match of the COMP-QTL and the *COMP* gene itself suggests a regulatory polymorphism to account for elevated serum levels in STR/ort mice and questions the robustness of serum COMP as a prognostic marker in human knee OA. The newly identified QTL associated with degenerative changes of the knee joints support the concept of OA resulting from a defective chondrocyte metabolism and/or altered apoptosis rate. However, we also discuss the unlikelihood of one QTL being responsible for OA in STR/ort mice and the inherent limitations of microsatellite analyses for complex genetic diseases.

Key words: Osteoarthritis, Genetics, Animal model, Microsatellite analysis.

Introduction

Osteoarthritis (OA) is the most common form of human joint disease and among the leading causes of disability throughout the world. Its hallmarks are degradation of the articular cartilage and remodelling of the subchondral bone leading to the functional impairment of the affected joints[1]. There is an increased recognition that OA results from active disease processes affecting the cartilage metabolism itself and chondrocyte apoptosis[2,3]. Furthermore, OA is a complex disease with multiple environmental and genetic factors contributing to the pathogenesis whereby the contribution of environment and genetics varies depending on the joint involved. While nodal hand OA shows the highest genetic influence, knee OA is more dependant on environmental factors with mechanical overload and trauma being the most prominent risk factors[4]. It is probably due to the heterogeneity of the disease at hand, the diversity of the human population and the variability of the environment that the genetics of OA are still poorly defined[5].

In order to further elucidate the genetics of OA we here turned to a mouse model which closely resembles human OA. The STR/ort mouse spontaneously develops degenerative changes of the knee joints including the loss of hyaline cartilage, osteophyte formation, calcification and ossification of cruciate ligaments and chondroid metaplasia[6]. By the age of 9 months, 85% of the male mice show these degenerative changes to varying degrees[7]. In addition, they become obese[8] and show elevated serum levels of the cartilage oligomeric matrix protein (COMP) (own unpublished results). The latter is particularly relevant as elevated serum COMP has also been described for human knee OA patients and has been suggested as a prognostic marker[9,10]. The goal of the present study was two-fold: to perform a genome wide microsatellite analysis to investigate the genetics of murine OA in the STR/ort mouse and to test STR/ort-specific phenotypic markers for their correlation with disease progression.

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Materials and methods

MICE

STR/ort mice were originally purchased from Harlan Winkelmann (Borchen, Germany) and subsequently bred in our animal facility under specific germfree conditions. C56BL/6 mice were purchased from Charles River (Sulzfeld, Germany). For the F2 generation, 15 pairs of grandparents (nine female STR/ort and nine male C57BL/6 and six female C57BL/6 and six male STR/ort mice) were mated. A total of 44 pairs of F1 brothers and sisters then gave rise to 276 male offspring. Of these 148 had STR/ort grandmothers and 128 had STR/ort grandfathers. Between one and three siblings were kept in one cage without environmental enrichments. This study was approved by the local state's animal care committee.

GENOTYPING

Genomic DNA used for genotyping the mice was isolated from 1 cm tail clips following standard isolation protocols[11]. Microsatellite analysis was performed on all 276 male F2 animals using 96 informative markers. These 96 microsatellites covered the mouse genome at a mean inter-marker distance of 11.6−18.75 cM for the different chromosomes. The protocol for the genotyping was as follows: genomic DNA (20 ng) was amplified in a final volume of 10 μl containing SAWADY *Taq* polymerase (0.25 U) (PeqLab, Erlangen, Germany), 1 mM $MgCl_2$, 0.02 mM deoxy nucleotide triphosphates (dNTP), primers (0.05 μM each) (Metabion, Martinsried, Germany), and 0.06 μM M13-IRD700 (Metabion, Martinsried, Germany) or M13-IRD800 (Sigma-Proligo, The Woodlands, TX, USA). Amplification conditions were as follows: 95°C for 10 min, followed by two cycles of 94°C for 30 s, 59°C for 1 min, 72°C for 1 min, followed by two cycles of 94°C for 30 s, 57°C for 1 min, 72°C for 1 min, then another 35 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min and a final extension at 72°C for 7 min. The reactions were performed using GeneAmp PCR System 9700 cycler (Applied Biosystems Inc., Foster City, CA, USA). The polymerase chain reaction (PCR) products were multiplexed (between two and 10 microsatellites at a time) and resolved on a CEQ 8800 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). The genotypes were scored independently by two authors.

PHENOTYPING

OA score

All F2 animals were sacrificed at 9 months of age. Both knees were prepared, decalcified and embedded into paraffin before thin sectioning in an anterior to posterior direction[12]. Haematoxylin and eosin stained sections were analyzed for osteoarthritic changes: grade 0, healthy cartilage; grade 0.5, irregularities of the cartilaginous surface; grade 1 and 1.5, small and intermediate fissural ulcerations; grade 2, limited loss of articular cartilage; grade 2.5, substantial loss of articular cartilage with minor areas of subchondral bone being exposed; grade 3, complete loss of articular cartilage with the subchondral bone being exposed (see Fig. 1). For each mouse, the medial and lateral femoral condyles, medial and lateral tibia plateaus and the surfaces of the patella−femoral joints of both knees were scored and in order to evaluate each joint surface at least once, between three and 10 sections (4 μm) per joint were analyzed and the highest scores observed were taken. For each mouse, an overall- and a lateral-tibia-joint-score were assessed. The former considered all joint surfaces and was calculated by dividing the sum of the individual scores by the number of joint surfaces analyzed. This calculation was necessitated by the fact that individual joint surfaces were missed because knees that were stiff could only be embedded in a bended fashion and because the sectioning was done in an anterior to posterior direction. We managed though to score all four tibiofemoral joint surfaces in 521 and both patellofemoral surfaces in 240 out of 552 joints analyzed. For the lateral-tibia-joint-score, the lateral tibial sides of the femorotibial compartments were analyzed and to rule out any dilution effects, only those mice were included where both knees could be evaluated. Again, both joint surfaces were summed up and divided by two. Thus the maximal scores per mouse were 3. Joints were scored independently and in a blinded fashion by two investigators.

Weight and serum COMP

At the time of death, mice were weighed, blood was taken from cardiac puncture and serum generated. COMP levels in the serum were quantified using the animal COMP enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (MD Biosciences, Zürich, Switzerland).

Linkage analysis

All linkage analyses have been performed using the imputational model in the R/qtl software package (R 2.4.1 and package qtl 1.05-2)[13,14]. The order of the loci was obtained from the mouse genome informatics database of the Jackson Laboratory (http://www.informatics.jax.org). OA score, serum COMP concentration and weight were taken as phenotypes. For the significant and suggestive linkage threshold values, we followed the guidelines for the permutation test of data (number of permutations = 1000) and significance levels of 95% ($P = 0.05$) and 90% ($P = 0.1$) were used to determine linkage.

Statistics

To screen for linkages between the various phenotypes, correlation tests were performed using InStat3 software (www.GraphPad.com). A P-value < 0.05 was considered significant.

Results

OA IN THE F2 PROGENY

In order to investigate the genetics of murine OA, an intercross between STR/ort and C57BL/6 mice was set up, an F2 generation bred and a genome wide microsatellite analysis performed on the F2 animals. The C57BL/6 strain was chosen because even though these mice do develop OA, the degenerative changes are milder than in STR/ort mice and tend to develop later[15]. Moreover, C57BL/6 mice carry the same H-2b haplotype as STR/ort and preclude any major histocompatibility complex (MHC) related effects, which have been implicated for human OA[16]. In detail, 276 male F2 animals plus STR/ort and C57BL/6 fathers were raised and analyzed at the age of 9 months for osteoarthritic changes of both knee joints (Fig. 1). First, an overall-score was calculated and here the median scores for the C57BL/6, F2 and STR/ort mice were 0.60, 1.67 and 0.954, respectively [Fig. 2(A)]. For the F2 animals, 2.7% of the tibial plateaus and 8.2% of the femoral condyles could not be evaluated, however, since they distributed evenly over all mice and joints, an overall-score seemed justified. Interestingly, the lateral sides of the knee joints showed greater cartilage damage than the medial ones: 41.3% of the lateral tibia plateaus and 22.0% of the lateral femoral condyles presented a score equal to or greater than 2 while it was only 15.3% for the medial tibia plateaus and 12.2% for the medial femoral condyles. Due to the anterior to posterior direction of sectioning, only 61% of the patellar and 54% of the femoral sides of the patella−femoral joints could be evaluated. Here, 26.7% of the patellar and 11.4% of the femoral sides showed scores equal to or greater than 2. In order to control for a possible averaging down of the highest scores in the overall-estimation, the lateral-tibia-joint-score representing the most severely affected joint surfaces was calculated. The comparison to the parental animals is shown in Fig. 2(B). The median scores were 1.125 for the C57BL/6 and 1.5 for both, the F2 and STR/ort animals.

Animals with an STR/ort grandmother did not differ in their OA scores compared to animals with an STR/ort grandfather indicating that the inheritance of OA is neither linked to mitochondrial DNA nor the Y-chromosome (data not shown).

SERUM COMP LEVELS IN THE F2 PROGENY

In search of an alternative phenotype to monitor osteoarthritic changes, serum COMP levels were determined for the parental and for 161 F2 animals. Again, the C57BL/6 and STR/ort mice represented the lower and upper ends of this phenotypic trait with medians of 0.79 and 1.24 U/L, respectively. The F2 animals range in between with a median of 0.86 U/L, an interquartile range of 0.69−1.11 U/L and lower and upper extremes of 0.08 and 2.08 U/L, respectively [Fig. 2(C)]. Interestingly, there was no correlation

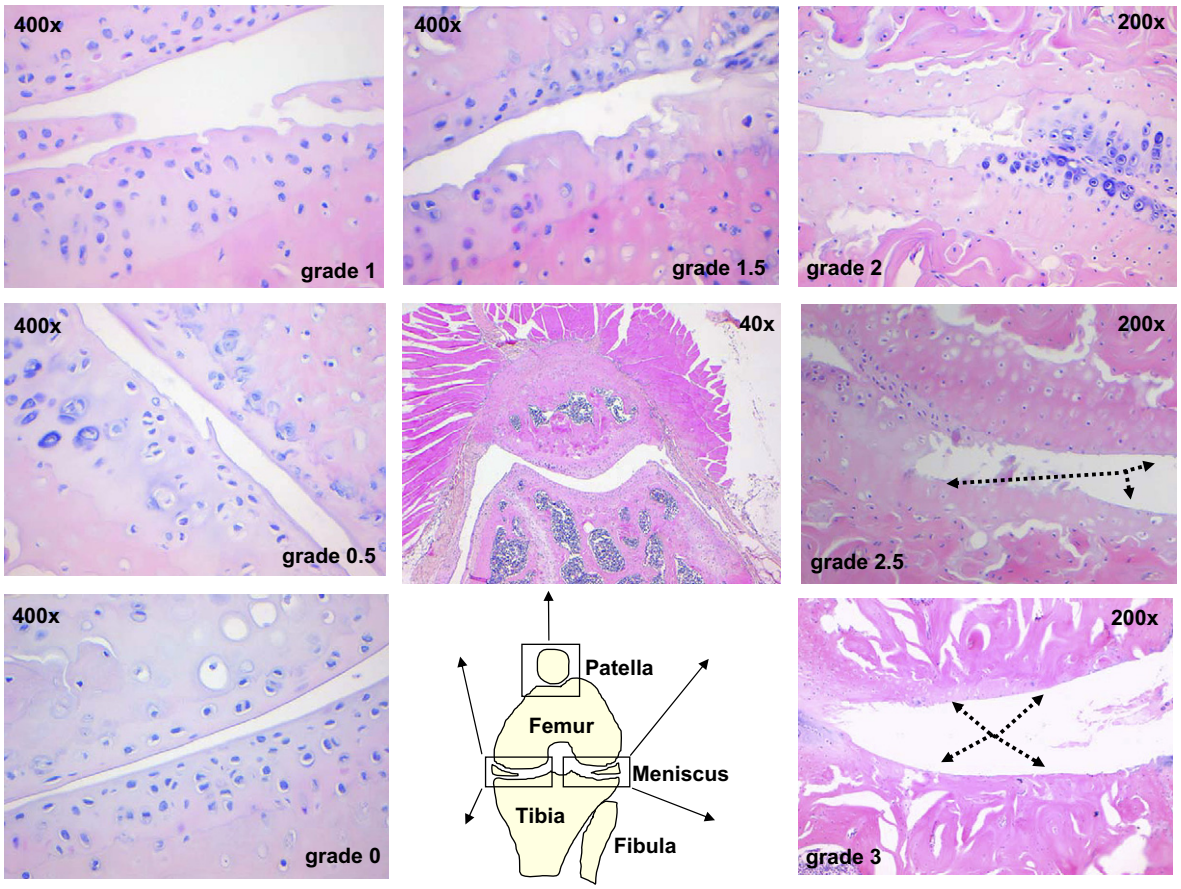

Fig. 1. The F2 progeny develops degenerative changes of the knee joints comparable to the parental STR/ort animals. Thin sections of the knee joints were stained with haematoxylin and eosin and were analyzed for osteoarthritic changes: grade 0, healthy cartilage; grade 0.5, irregularities of the cartilaginous surface; grades 1 and 1.5, small and intermediate fissural ulcerations; grade 2, limited loss of articular cartilage; grade 2.5, substantial loss of articular cartilage with areas of subchondral bone being exposed; grade 3, complete loss of articular cartilage with the subchondral bone being exposed. The lower centre panel gives a schematic overview of the knee joint with insets indicating the joint surfaces which were evaluated. The centre panel shows a patella–femoral joint, the panels to the left, to the right and to the top show examples of grades 0–3. Dashed arrows point to exposed bone.

between the OA score and the serum concentration of COMP indicating that both are independent phenotypic traits in STR/ort mice and their progeny.

WEIGHT IN THE F2 PROGENY

As STR/ort mice tend to become obese with age we were interested in a potential correlation between weight and osteoarthritic changes of the knee joints. To that end we weighed the mice at the time when osteoarthritic changes were analyzed and tested for a correlation between weight and OA score. The median weight of the F2 progeny was 46.9 g with an interquartile range of 42.7–50.9 g and lower and upper extremes of 23.9 and 65.2 g, respectively. The weight of the STR/ort mice was comparable with a median of 43.5 g with an interquartile range of 41.5–49.3 g and lower and upper extremes of 38.3 and 52.7 g, respectively. In contrast, C57BL/6 mice weighed the least with a median of 35.6 g, an interquartile range of 33.5–45.1 g and lower and upper extremes were 33.0 and 53.0 g, respectively [Fig. 2(D)]. Interestingly, correlation analyses indicated a trend towards a negative linkage between weight and OA ($P = 0.0611$) and indeed, mice with the highest OA scores tended to have the lowest weights (data not shown). As these lowest-weight mice appeared cachectic we do not believe that the weight reduction resulted from an increased physical activity. We rather believe that obesity in STR/ort mice is neither a cause for nor a consequence of OA but rather an independent trait.

QUANTITATIVE TRAIT LOCI (QTL) ASSOCIATED WITH BODY WEIGHT

To screen for weight-associated QTL, we performed linkage analyses for this trait in the F2 progeny. The single-QTL genome scan identified two QTL on chromosomes 1 and 18 with logarithm of odds (LOD) scores of 4.36 and 6.39, respectively. Evidences of linkage segregated with the positions 46.5 and 31.5 cM [Fig. 3(A)]. The two-dimensional, two-QTL genome scan yielded three pairs of interacting QTL, the first one of which comprised the two loci on chromosomes 1 and 18 identified *via* the single-QTL scan. The LOD score was 12.88 and passed the 0.99 significance level. The other two pairs comprised again either of the two loci on chromosomes 1 and 18 plus an additional QTL on chromosome 11 at position 72.4 cM [Fig. 3(B)]. The LOD scores were 8.38 (passing the 0.90 significance level) and 11.94 (passing the 0.99 significance level). In summary, we identified three weight-associated QTL which

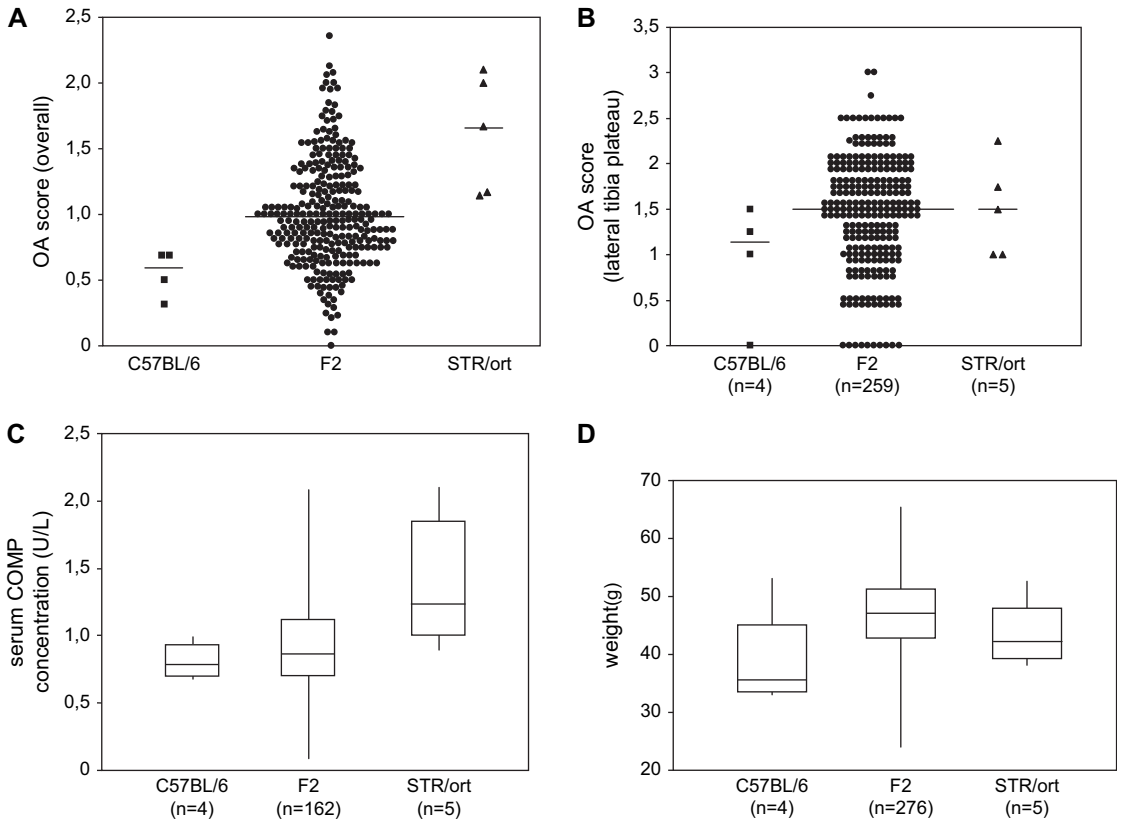

Fig. 2. Degenerative changes of the knee joints, serum COMP concentrations and body weight in the F2 progeny suggest Mendelian inheritance. The dot plots illustrate the distribution of OA scores (A and B), where each symbol represents a single mouse and medians are indicated by vertical bars. Box plots show serum COMP (C) and body weight (D) among parental C57BL/6 and STR/ort as well as F2 mice. Data are given as medians and interquartile ranges as well as lower and upper extremes. The number of animals analyzed is indicated (*n*).

seem to interact with each other in order to promote body weight in STR/ort mice.

QTL ASSOCIATED WITH SERUM COMP LEVELS

To screen for QTL associated with serum COMP concentrations, single- and two-QTL genome scans were performed. While the two-QTL scan produced no results, the single-QTL scan identified one locus on chromosome 8 at 33.5 cM. The LOD score was 10.0 and passed the 0.99 significance level [Fig. 4(A)].

QTL ASSOCIATED WITH OA

When screening for OA-associated QTL performing the single-QTL genome scan on the overall-score, one locus on chromosome 8 at 21.3 cM emerged. This linkage reached an LOD score of 3.3 and passed the 0.90 significance level [Fig. 4(B)]. No significant linkage emerged performing the single-QTL genome scan using the lateral-tibia-joint-score. Table I summarizes all the QTL identified and associated with phenotypic traits of STR/ort mice and gives the chromosomal locations, LOD scores and significance levels.

Discussion

This is the first microsatellite analysis elucidating the genetics of OA in a spontaneous animal model. Several unexpected findings emerge from this study: the first one is that the degenerative changes observed in F2 animals show characteristics of either parental strain like the STR/ort-specific severe damage of both, the tibiofemoral and the patellofemoral compartments of the knee joints[6,7] and the concentration of more severe damage at the lateral sides of the tibiofemoral joints reminiscent of the C57BL/6 strain[15]. As of yet we cannot explain how these degenerative changes observed in the F2 generation are inherited and whether there are aspects to the disease, which are secondary to musculoskeletal abnormalities. We therefore decided on two different scoring systems to evaluate OA in individual animals, the overall-score considering all joint surfaces and the lateral-tibia-joint-score considering the most severely affected joint surface in the F2 generation. For the overall-score, the median of the F2 generation lies between both the parental strains indicating Mendelian inheritance [Fig. 2(A)]. In contrast, the median lateral-tibia-joint-score for the F2 generation is identical to the STR/ort animals reflecting the fact that in the STR/ort strain it is the medial tibial plateaus which are most severely affected. However, we here analyzed only a small number of C57BL/6 and STR/ort animals because both strains have been characterized extensively, before[6,7,15]. Consequently, the inherent inter-individual variability of mouse models carries more weight which is why in our comparison the F2 animals exceed both parental strains in the lower and upper extremes and in all three phenotypic markers weight, serum COMP levels and OA score (Fig. 2).

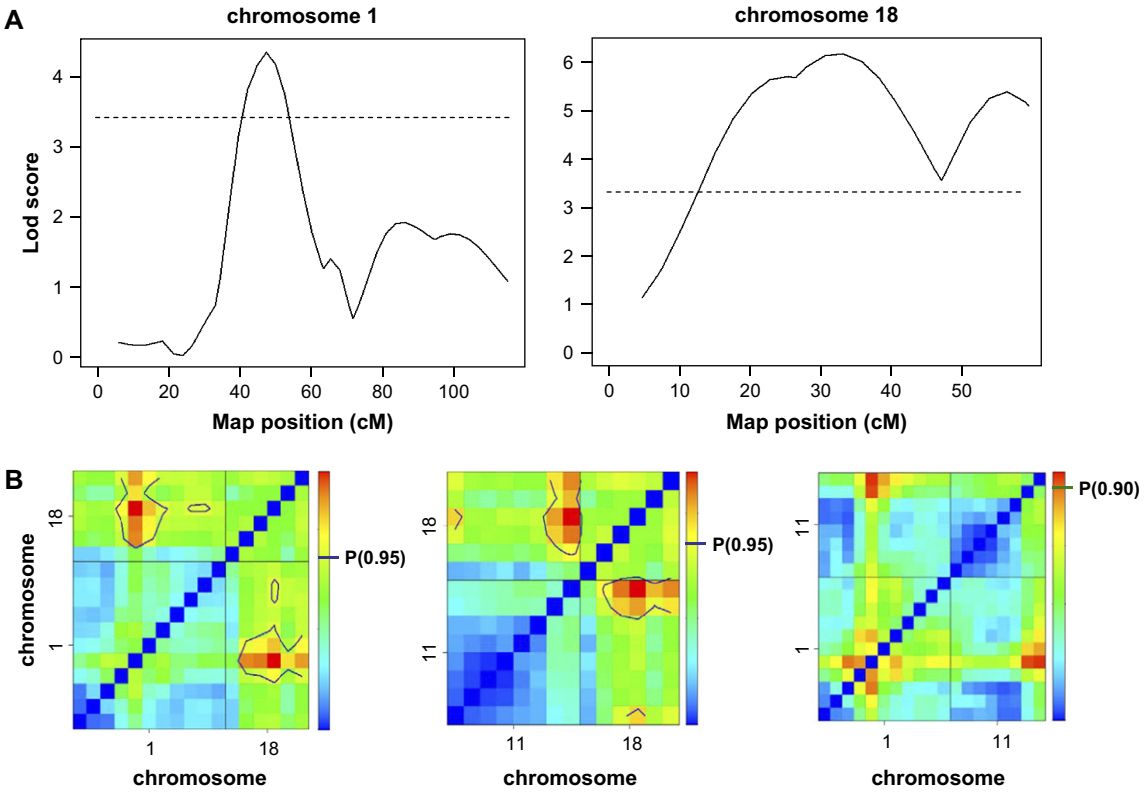

Fig. 3. Linkage analyses reveal three weight-associated QTL on chromosomes 1, 11 and 18. (A) The single-QTL analysis identified two weight-QTL on chromosomes 1 and 18, at 46.5 and 34 cM, respectively. Significance levels according to 1000 permutation tests were $P(0.90) = 3.1$ and $P(0.95) = 3.43$. The dotted lines indicate the 0.95 significance level. (B) The two-QTL analysis identified three pairs of interacting weight-QTL, chromosome 1 (46.5 cM) with chromosome 11 (72.4 cM), chromosome 1 (46.5 cM) with chromosome 18 (34 cM) and chromosome 11 (72.4 cM) with chromosome 18 (34 cM). The interaction graphs each consider the full model (two QTL plus interaction) and the significance levels according to 1000 permutation tests were $P(0.90) = 7.98$ and $P(0.95) = 8.52$, the latter ones are indicated by blue lines within the graph.

The second unexpected finding is that in STR/ort mice, degenerative changes of the knee joints – independent of overall- or lateral-tibia-joint-score – neither correlate with obesity nor with elevated serum COMP but rather seem to be independent traits. This is different from the human situation where obesity is often associated with knee OA and is therefore considered a risk factor[17]. The genetic linkage analyses revealed three chromosomal locations associated with weight in STR/ort mice and indeed, all three harbour genes or QTL related to the fatty acid metabolism. Chromosome 1 at position 48 cM encodes the high density lipoprotein (HDL)-QTL 41 (Hdlq41), chromosome 11 at positions 70 and 72 cM encodes the triglyceride (Trigl)-QTL 2 (Triglq2) and the fatty acid synthase (Fasn), respectively, and chromosome 18 at position 32 cM encodes Hdl40, a QTL associated with the HDL level (www.jax.org). All four have previously been implicated in regulating body weight in mice[18–20]. Note that the weight-QTL on chromosome 11 by itself does not pass any significance threshold yet yields an extremely significant LOD score when analyzed for an interaction with the weight-QTL on chromosome 18.

The lack of a correlation between elevated serum COMP and osteoarthritic changes of the knee joints in mice is particularly interesting because again, the opposite has been suggested for human knee OA. Here, serum COMP has even been discussed as a prognostic marker of disease progression[9]. In STR/ort mice, one QTL associated highly significantly with serum COMP and this QTL was located on chromosome 8 at position 33.5 cM. In fact, this linkage could hardly be improved even if all 276 animals were analyzed for serum COMP levels. Most strikingly, the *COMP* gene itself is located at position 33 cM and we consider it likely that regulatory polymorphisms within the gene are responsible for varying COMP levels in the serum. Indeed, there are several single nucleotide polymorphism (SNP)s in the promoter region which could impact on gene regulation, among them one at position 239 within a previously described transcriptional repressor (www.ensembl.org)[21]. It will be necessary to determine whether elevated serum COMP in human OA patients is a true diagnostic marker or – as in STR/ort mice – an epiphenomenon resulting from an OA-independent SNP in the *COMP* gene itself.

The most striking result from the present microsatellite analysis is the fact that only one OA-associated QTL emerged from the overall-score and passed the 0.90 significance level. There are several explanations for this unexpectedly lean result, among them the possibility that our choice of microsatellite markers was suboptimal or that the inter-marker distances were too large. However, our identification of convincing weight- and serum COMP-QTL argues against this possibility. Alternatively, OA in STR/ort mice may be a monogenic disease with only a weak genetic association. However, the high penetrance of OA in the F2 generation [Fig. 2(A) and (B)] rather argues for a strong association and if indeed monogenic, we expect LOD scores comparable to the COMP-QTL. So why did we fail to

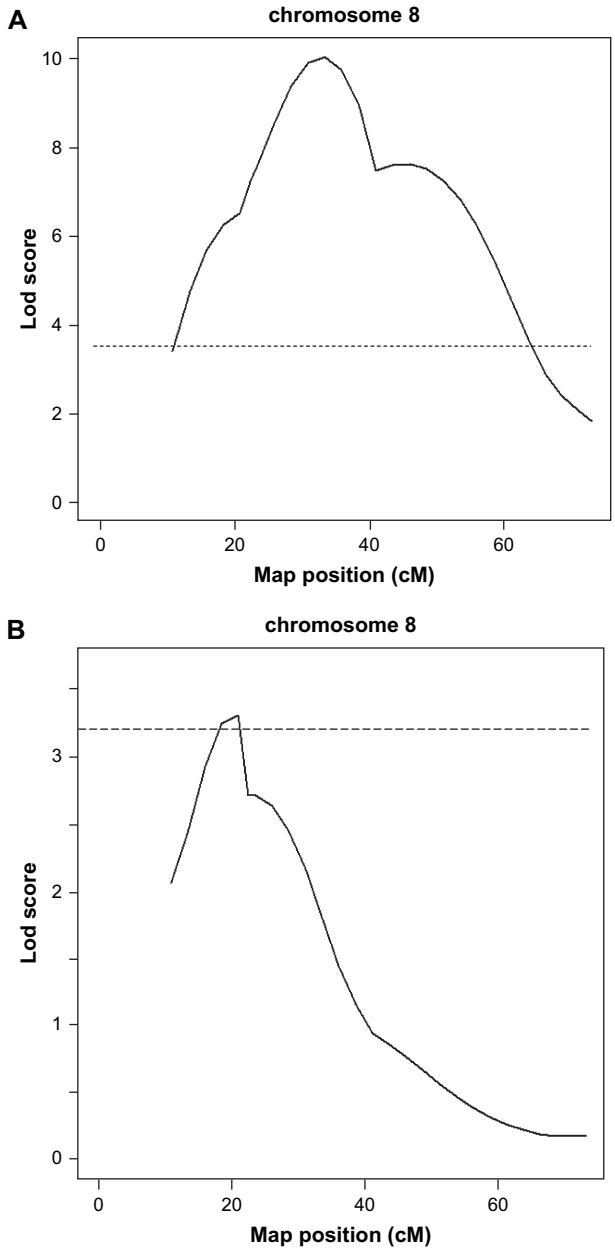

Fig. 4. Linkage analyses reveal one COMP-QTL and one OA-QTL, both on chromosome 8. (A) Single-QTL analysis identified one COMP-QTL on chromosome 8 at 33.5 cM (A). The significance levels according to 1000 permutation tests were $P(0.90) = 3.21$ and $P(0.95) = 3.52$ and the dotted line indicates the 0.95 significance level. (B) Single-QTL analysis identified one OA-QTL on chromosome 8 at 21.3 cM. Significance levels according to 1000 permutation tests were $P(0.90) = 3.19$ and $P(0.95) = 3.56$. The dotted lines indicate the 0.90 significance level.

identify strong OA-QTL in the STR/ort mouse? We favour a third possibility which implies that OA in mice – just like in humans – is a multigenic disease and requires the interaction of multiple gene loci in order to trigger the pathogenesis. Each individual gene may exert only a modest effect and will therefore fail to pass the significance threshold in the single-QTL analysis, which is what happened to the weight-QTL on chromosome 11. However, in contrast to the weight analysis, the two-QTL scan for knee OA-associated

Table I
Summary of QTL associated with phenotypic traits of STR/ort mice

Trait	Chromosome	Position	LOD*
Scan 1†			
Weight	1	46.5	4.36***
Weight	18	31.5	6.39***
Serum COMP	8	33.5	10.0***
OA (overall-score)	8	21.3	3.3*
Scan 2‡			
Weight	1:11	46.5:72.4	8.38*
Weight	1:18	46.5:34.0	12.88***
Weight	11:18	72.4:34.0	11.94***

Significance levels are ***P(0.99), **P(0.95) and *P(0.90) according to 1000 permutation tests.
*LOD scores calculated with R/qtl.
†Single-QTL scan.
‡Two-QTL scan.

gene loci did not produce an additional QTL. What is required here are multiple-QTL analyses which unfortunately, are not available as of today. These computational limitations may also explain the lack of coherence among the data on human OA where genetic and environmental backgrounds decide whether individual genes act by themselves or as partners in a set of two or three or even more genes.

The fact that no OA-associated QTL emerged from the lateral-tibia-joint-score suggests that the overall-score indeed corroborates the cartilage damage characteristic of OA rather than averaging down the highest scores. It also points out that a detailed histological sectioning combined with a global approach is an absolute requirement for our analysis.

Figure 5 summarizes the microsatellite markers used, their relative positions in the genome and the QTL identified for weight, elevated COMP and knee OA in the STR/ort mouse. We consider the OA-associated QTL identified in position 21.3 cM on chromosome 8 only the tip of the iceberg, yet this chromosomal region already suggests a number of interesting candidate genes each of which holds the potential to impact on cartilage metabolism and apoptosis (www.jax.org). In detail, the 6 Mb surrounding the position 21.3 cM harbour about five QTL, 18 predicted and 15 identified genes, among them *DUSP4*, a phosphatase which is involved in the mitogen activated protein kinase (MAPK) signal transduction pathway and thus potentially exerts a plethora of effects on apoptosis, cell cycle and cell senescence[22]. *DUSP4* is particularly interesting due to its close relatedness to *DUSP2*, a disease-associated gene in human OA[23]. Another gene located on this QTL is *TNKS* encoding a tankyrase, a polyADP ribose polymerase controlling longevity *via* regulation of telomere stability and/or post-translational protein modifications[24]. Additional genes encoded on the OA-associated QTL and possibly involved in the regulation of transcription or protein expression are *CNOT7* encoding a transcriptional activator, *ZDHHC2* encoding a zinc finger and *THEX1* encoding an exonuclease which in *Caenorhabditis elegans* has been implicated in degradation of siRNA[25]. *FGF20* encodes a fibroblast growth factor and not only do members of this family play important roles in proliferation, differentiation and apoptosis, Fgf-20 in particular has been shown to be expressed in the developing mouse limbs[26].

By increasing the density of microsatellite markers on chromosome 8 we hope to narrow down the number of candidate genes even further before testing individual genes in

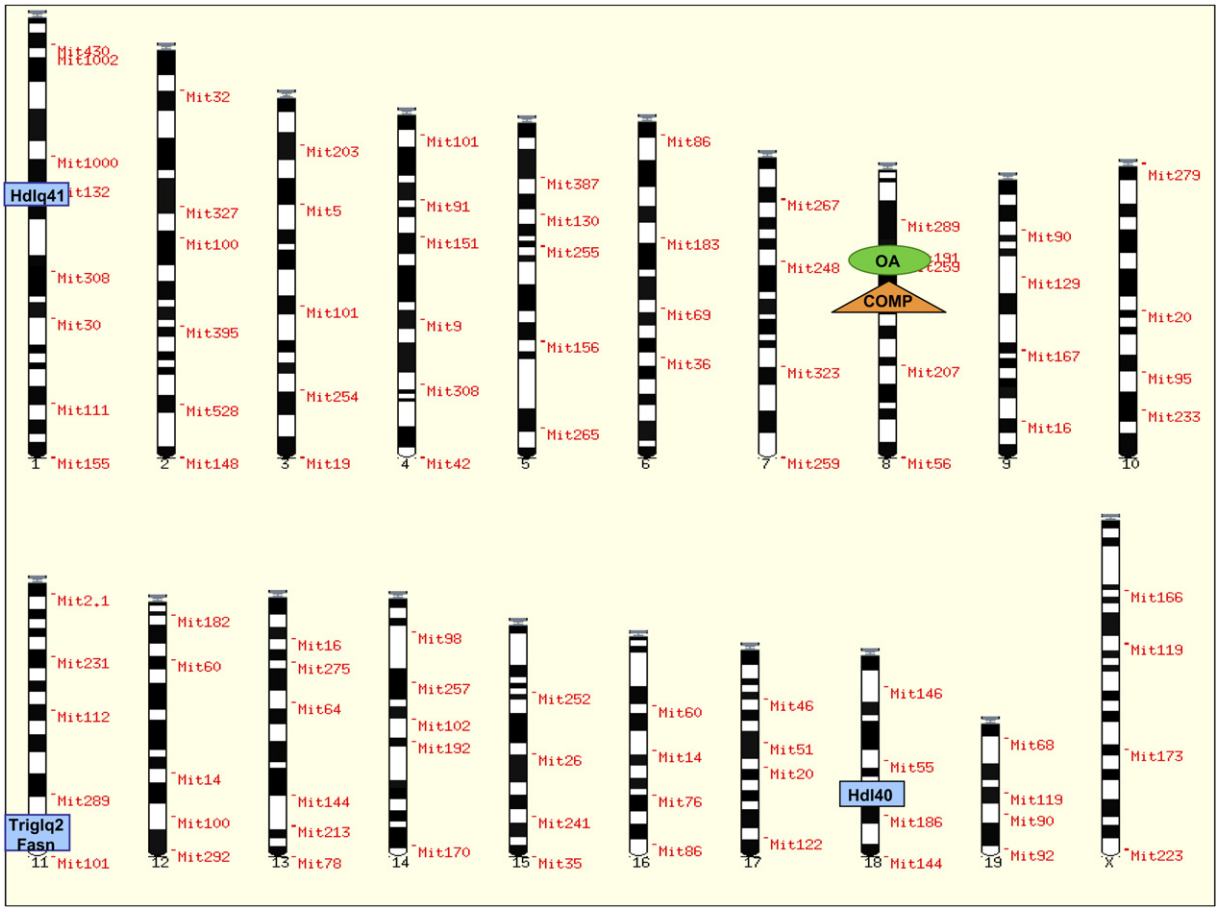

Fig. 5. Position of the weight-, COMP- and OA-QTL in the murine genome. The karyogram summarizes the positions of the microsatellites used and our newly identified QTL. The blue boxes depict the weight-QTL and indicate for the same positions previously described HDL- and Trigl-associated QTL and the gene encoding Fasn. The orange triangle describes the position of the COMP-QTL which is congruent with the position of the *COMP* gene itself. The green oval symbolizes the OA-QTL.

in vitro assays for their potential to manipulate chondrocytes. However, this will only provide us with one disease-associated gene and – as mentioned above – we doubt that OA in the mouse is a monogenic disease. We will therefore have to develop novel strategies to identify all the different QTL involved in the pathogenesis of murine OA before we can transfer our results to human OA.

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